In the Claims:

Page 16, above claim 1, insert the following:

What is claimed is:

In the Abstract:

Page 17, line 1, replace the heading with the following new heading

ABSTRACT OF THE DISCLOSURE

In the Sequence Listing:

Please replace the Sequence Listing of record with the attached substitute Sequence Listing consisting of SEQ ID Nos. 1-12.

REMARKS

The foregoing amendments are presented to place the application in compliance with the sequence rules under 37 CFR 1.821-1.825.

Applicants have submitted a Sequence Listing in both paper and computer readable form as required by 37 C.F.R. 1.821(c) and (e). Amendments directing its entry into the specification have also been incorporated herein. The content of the paper and computer readable copies are the same and no new matter has been added.

Additional amendments to the specification have also been effected to put the specification in better form under U.S. practice. Specifically, the specification headings have been amended in

conformance with U.S. practice. Also, the additional sequences disclosed in the specification and Figure 3 have been identified and labeled as required under U.S. practice.

With regard to the Notice also requesting that an executed Oath and Declaration of the Inventors needs to be submitted, Applicants wish to note that an executed Oath and Declaration was submitted on January 23, 2002. A copy of the submitted executed Declaration is enclosed herewith along with the cover letter (indicating the filing of the executed Declaration). Applicants respectfully request that the Patent Office review the application papers to ensure that the executed Declaration is present in the file.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "**Version with markings to show changes made.**"

In view of the foregoing, it is believed that each requirement set forth in the Notice has been satisfied, and that the application is now in compliance with the sequence rules under 37 CFR 1.821-1.825. Accordingly, favorable examination on the merits is respectfully requested.

Respectfully submitted,

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Version with Markings to Show Changes Made

~~DESCRIPTION~~

Plant Thermogenic Genes and Proteins

Background of the Invention

1. Technical Field of the Invention

The present invention relates to plant thermogenic genes and proteins. More particularly, the invention relates to thermpogenic genes derived from a skunk cabbage (*Symplocarpus foetidus*) and gene products (proteins). Those genes and proteins are useful in breeding of cold-avoidance plants, medical treatment of diabetes mellitus or obesity, or development of novel thermogenic bio-materials.

2. Description of the Related Art

~~Background~~

Stresses due to low temperatures, droughts and salinity are common harmful environmental factors that terrestrial plants encounter. Among these stresses, it has been considered that cellular injury due to the low temperature is the most important factor which restricts productivity of crops (Levitt, 1980). To resist the low temperature stress, cold-hardy plants such as wheat or rye have a variety of physiological and metabolic responses which lead to cold acclimation (Sakai and Larcher, 1987; Steponkus, 1984; Thomashow, 1998; Uemura and Steponkus, 1997). In contrast, it is known that some plants including skunk cabbage have a specialized system by which the plants generate heat to avoid freezing (Knutson, 1974; Nagy et al., 1972; Schneider and Buchenen, 1980).

The temperature of the flower in the spadix of skunk cabbage, which flowers in early spring, has been known to maintain its temperature at higher

the C-terminal region and increases by free fatty acids (Jezek et al., 1998; Lin and Klingenberg, 1982; Katiyar and Shrago, 1989; Rial et al., 1983; Sluse et al., 1998).

On the contrary, 2 cDNAs encoding UCP-like proteins of plant origin were isolated from potato (StUCP: Laloi et al., 1997) and from *Arabidopsis* (AtPUMP: Maia et al., 1998). Since the expression of StUCP was mainly detected in the flower and the fruit, it has been postulated that StUCP may concern respiration during flowering and maturation of the fruit together with the AOX activity (Laloi et al., 1997).

Potato and *Arabidopsis* have been considered to be non-thermogenic plants. However, the expression of StUCP and AtPUMP have been induced by low temperature. Therefore, it has been suggested that these genes are involved in ~~the~~ heat production (Laloi et al., 1997; Maia et al., 1998).

In the thermogenic plants such as skunk cabbage, however, UCP-mediated thermoigenic mechanisms have not yet been identified.

The purpose of the invention of this application is to provide unidentified novel UCP genes derived from a thermogenic plant, skunk cabbage.

The additional purpose of this application is to provide skunk cabbage UCPs which are expression products of the novel genes.

Summary of the Disclosure of Invention

The invention provides thermogenic genes derived from skunk cabbage, i.e., gene SfUCPa of which cDNA comprises the base sequence of SEQ ID NO: 1, and gene SfUCPb of which cDNA comprises the base sequence of SEQ ID NO: 3.

Moreover, the invention provides thermogenic proteins, i.e., protein SfUCPA expressed from SfUCPa, which comprises the amino acid sequence of SEQ ID NO: 2, and protein SfUCPB expressed from SfUCPb, which comprises the amino acid sequence of SEQ ID NO: 4.

In addition, the invention provides cDNA having the base sequence of SEQ ID NO: 1 or a partial sequence thereof, and cDNA having the base sequence of SEQ ID NO: 3 or a partial sequence thereof.

Brief Description of Drawings

Fig. 1 shows the change of the temperature of the spadix in skunk cabbage and that of ambient temperature with a lapse of time.

Fig. 2 shows the results of northern blotting, indicating the expression profile of SfUCPa (A) and SfUCPb (B) in the spadix and leaf of skunk cabbage at room temperature (RT) and during cold treatment (4°C for 3 days). The lower figures respectively show the results of ethidium bromide staining of non-decomposed rRNA.

Fig. 3 compares the alignment of amino acid sequences of SfUCPA and SfUCPB, together with potato UCP (StUCP), *Arabidopsis* UCP (AtPUMP) and human UCP. The asterisks (*) attached under the sequences indicate the same amino acid sequence, and the dot (.) indicates the conservative change in all of the sequences. The boldface indicates the same sequence between SfUCPA and SfUCPB. The gap introduced to optimize the sequence alignment is indicated by a dash (-). The alignment was made using a CLUSTAL W program. The characteristic domains of energy transfer proteins typical of mitochondria are

surrounded by a square. The shaded bars (I~VI) above the upper sequence show estimated transmembrane domains.

Fig. 4 shows a hydrophobic plot of SfUCPA. The vertical axis indicates the degree of hydrophobicity and the estimated transmembrane domains are indicated by TM1 to TM6.

Fig. 5 shows a diagrammatic illustration of SfUCPA topology in the mitochondria membrane.

Fig. 6 shows a hydrophobic plot of SfUCPB. The vertical axis indicates the degree of hydrophobicity and the estimated transmembrane domains are indicated by TM1 to TM4 and TM6.

Fig. 7 shows a diagrammatic illustration of SfUCPB topology in the mitochondria membrane.

Fig. 8 shows the results of *in vitro* translation using respective cDNAs of the genes SfUCPa and SfUCPb as templates. (-) indicates a control, S a sense RNA, and AS an antisense RNA. The asterisk (*) indicates a non-specific product and the empty circle denotes the position of a low molecular translated artificial product synthesized from a small ORF.

Description of the Preferred Embodiments
~~**Best Mode for Carrying Out the Invention**~~

In the gene SfUCPa of the present invention, its cDNA has the base sequence of SEQ ID NO: 1 and encodes the protein SfUCPA having the amino acid sequence of SEQ ID NO: 2, of which the estimated molecular weight is 32.6 kDa. In the gene SfUCPb of the present invention, its cDNA (SEQ ID NO: 3) encodes

many include mammal cultured cells such as monkey renal cell COS7, Chinese hamster ovarian cell CHO, etc., budding yeast, fission yeast, silkworm cell, Xenopus egg cell, and the like are commonly used, but not limited thereto. In order to introduce the expression vector into eucaryotic cells, a known method such as electroporation, calcium phosphate method, liposome method, DEAE dextran method, and the like can be utilized.

After expression of the proteins in procaryotic cells or eucaryotic cells according to the aforementioned method, the desired proteins are isolated and purified from the culture in the known combined procedures for separation. For example, treatment with a denaturant (e.g., urea) or surface activator, ultrasonication, digestion with enzymes, salting-out or solvent precipitation, dialysis, centrifugation, ultrafiltration, gel filtration, SDS-PAGE, isoelectric focusing, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, reverse phase chromatography, and the like are involved.

The proteins of the invention, SfUCPA and SfUCPB, also include peptide fragments (5 amino acids or more) involving the optional partial amino acid sequences of SEQ ID NOS: 2 and 4. In addition, the proteins of the invention also include fusion proteins with other optional proteins.

The following examples serve to illustrate the invention of this application specifically in more detail, but are not intended to limit the scope of the invention.

Example 1: Cloning of cDNA

The total RNA was extracted from the spadix of skunk cabbage (*Symplocarpus foetidus*) and the complete RNA was determined on 1.0% agarose gel electrophoresis (Ito et al., 1999). Using a mRNA isolation kit (Pharmacia), a

clone associated with the UCP gene family was isolated from the purified poly(A)+RNA by RT-PCR. The first strand cDNA was prepared by annealing 20 pmol of cDNA primed primer (5'-TTTTTTTTTTTTTTTTTTTTTT-3') (SEQ ID NO. 10) into poly(A)+RNA (0.1μg), followed by extension with 10 units of reverse transcriptase (New England Biolab) at 37°C for 30 minutes in 20μl of 1×RT buffer containing 10mM 1,4-dithiothreitol and 0.5mM dNTP. The composition of the reaction solution is as follows.

- 10mM Tris-HCl (pH 8.0);
- 50mM KCl;
- 1.5mM $MgCl_2$;
- 4mM dNTP;
- 0.2 unit of EX Taq polymerase (Takara); and
- 10pmol of two degenerate primers corresponding to the conserved amino acid sequence of the UCP family:

ZF1 (5'-CCIYTIGAYACIGCIAAR-3') (SEQ ID NO. 11)

ZR1 (5'-ACWTTCCAISYICCIAWIC-3') (SEQ ID NO. 12)

PCR cycle was carried out as follows.

(94°C: 0.5 minute; 50°C: 1 minute; 72°C: 1 minute)×35

Among the PCR products obtained in the above method, the amino acid sequence estimated from the sequence of about 0.8kb cDNA fragment had very high homology to one of the reading frame sequences of the UCP gene family. This fragment, accordingly, was cloned into T-vector (clone p2-1) and used as a probe for library screening.

cDNA (5μg) prepared from the spadix was inserted into λgt11 phage according to the known method (Sambrook et al., 1989) to construct a cDNA library. From this library, 8 clones positive to the above-described probes were isolated and sub-cloned into the pBluescript SK plasmid (Stratagene). From

linearized, on which a sense- or anti-sense RNA was transcribed with T7 RNA polymerase or T3 RNA polymerase according to the protocol of MAXICRIPT transcription kit (Ambion). An equal amount of RNA (4μg) was provided for in vitro translation reaction using a wheat germ extract (Promega) in the presence of ^{35}S-methionine (Amersham). The translation product was analyzed by SDS-PAGE. The gel was fixed, incubated in Amplify (Amersham), then dried, and fluorometrically analyzed.

As a result, it was confirmed that, as shown in Fig. 8, the initiation codon and the stop codon of cDNA isolated in Example 1 functioned successfully since a protein having an expected molecular weight was produced from any of cDNAs only when the sense RNA was used as a template.

Industrial Applicability

As described previously, this application provides novel thermogenic genes SfUCPa and SfUCPb as well as their gene products, i.e., thermogenic proteins SfUCPA and SfUCPB, derived from skunk cabbage (*Symplocarpus foetidus*), and cDNAs used for gene engineering mass production of these proteins. These genes and proteins allow development of low temperature-tolerant plants, development of drugs or methods for treatment of diabetes mellitus or obesity, or development of novel heat generating materials from plants.

References

Berthold and Siedow (1993) Plant Physiol. 101, 113-119.

Boss et al. (1997) FEBS Lett. 408, 39-42.

Fleury et al. (1997) Nature Genetics 15, 269-272.

CLAIMS

What is claimed is:

1. A thermogenic gene SfUCPa derived from skunk cabbage, of which cDNA comprises the base sequence of SEQ ID NO: 1.

2. A thermogenic gene SfUCPb derived from skunk cabbage, of which cDNA comprises the base sequence of SEQ ID NO: 3.

3. A thermogenic protein SfUCPA expressed from the gene SfUCPa of Claim 1, which comprises the amino acid sequence of SEQ ID NO: 2.

4. A Thermogenic protein SfUCPB expressed from the gene SfUCPb of Claim 2, which comprises the amino acid sequence of SEQ ID NO: 4.

5. A DNA fragment comprising the base sequence of SEQ ID NO: 1 or a partial sequence thereof.

6. A DNA fragment comprising the base sequence of SEQ ID NO 3: or a partial sequence thereof.

ABSTRACT OF THE DISCLOSURE

The inventions of this application include thermogenic genes named SfUCPa and SfUCPb which are derived from skunk cabbage. cDNA of each gene comprises the base sequence of SEQ ID NO: 1 and 3, respectively. Thermogenic proteins, SfUCPA and SfUCPB, are expressed from genes SfUCPa and SfUCPb, comprises the amino acid sequence of SEQ ID NO: 2 and 4.